REGULATION OF THE RELEASE OF CHROMATOPHOROTROPIC NEUROHORMONES FROM THE ISOLATED EYESTALK OF THE FIDDLER CRAB, *UCA PUGILATOR*

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ABSTRACT

Electrical stimulation of the isolated eyestalk of *Uca pugilator* induces the release of several peptides which affect epidermal chromatophores. Thresholds for release of these peptides were different, that for red pigment concentrating hormone (RPCH) being lowest, for black pigment dispersing hormone (BPDH) highest, and for black pigment concentrating hormone (BPCH) intermediate, but no red pigment dispersing hormone (RPDH) was detected at any voltage. Neurotransmitters, known to be present in crustacean central nervous systems, induced chromatophore dose dependent responses. Norepinephrine induced BPDH release, and dopamine induced both RPCH and BPCH release.

INTRODUCTION

Studies of the regulation of the release of peptide hormones from neuroendocrine cells have focused recently on the roles of neurotransmitters which inhibit or stimulate such release (Krulich, 1979; Moss, 1979). However, these studies have often resulted in conflicting observations due to dosage differences (Kordon *et al.,* 1981), feedback mechanisms (Krulich, 1979; Hökfelt *et al.,* 1980), or complex responses of the intact central nervous system (Fernlund *et al.,* 1980). Furthermore, simple implantation of high doses of potent neurotransmitters cannot distinguish between physiological and pharmacological responses (McKelvy *et al.,* 1980). For these reasons the trend has been to work with central nervous system fragments, hemisected pituitaries, isolated endocrine glands, or endocrine cells in culture (Krulich, 1979; Eiden and Brownstein, 1981; O'Donohue and Dorsa, 1982).

The endocrine control of pigment migration in chromatophores of crustaceans offers a sensitive tool for the investigation of neurotransmitters in endocrine regulation (Fingerman and Fingerman, 1977a, b). Peptidergic pigment dispersing and pigment concentrating chromatophorotropic hormones have been identified. A major site of synthesis of these peptides is the cell bodies of neuroendocrine cells in the medulla terminalis X-organ located within the eyestalk of most crustaceans. The axon terminals of these neuroendocrine cells comprise the sinus gland, the neurohemal organ, from which these neuroendocrines are released (Kleinholz and Keller, 1979; Cooke and Sullivan, 1983). Bioassays of these hormones can be performed in whole crabs, or isolated tissues, with both target cell specificity and picomolar sensitivity (Fingerman, 1973; Cooke *et al.,* 1977; Fingerman *et al.,* 1981a; Jaffe *et al.,* 1982; Riehm and Rao,

1982). Cooke *et al.* (1977) reported that the isolated sinus gland releases red pigment concentrating hormone (RPCH) in a voltage dependent, Ca^{++} sensitive manner. However, several other endocrines are also stored in the sinus gland and are probably released simultaneously with RPCH when such *in vitro* experiments are performed.

Rao and Fingerman (1970, 1975), Fingerman and Fingerman (1975; 1977a, b), Fingerman *et al.* (1981a, b), and Hanumante and Fingerman (1981a, b; 1982a, b) have reported that various neurotransmitters injected into fiddler crabs can induce specific endocrine mediated chromatophore responses. These authors suggested that the neurotransmitters trigger the release of specific chromatophorotropic hormones. The neurotransmitters have no effect when applied directly to the chromatophores in isolated legs. Briefly, these investigators found that norepinephrine (NE) indirectly induces black pigment dispersion, 5-hydroxytryptamine indirectly induces red pigment dispersion, and dopamine (DA) indirectly induces red pigment concentration. These observations are the only substantial evidence that neurotransmitters affect the release of arthropod neuroendocrines, despite a large literature on insect endocrinology (Gilbert *et al.,* 1980; Riddiford, 1980; Orchard and Loughton, 1981; Orchard *et al.,* 1982).

We designed experiments to learn whether the neurotransmitters NE and DA can affect endocrine release from the isolated eyestalk complex *in vitro.* In addition, the composition and biological activity of perfusates of isolated eyestalks which were electrically stimulated were determined.

Materials and Methods

Fiddler crabs, *Uca pugilator,* were obtained from the Gulf Specimen Supply Company, Panacea, Florida, and kept in a closed circulating sea water system until use (22°C; LD 12:12), lights on at 0800 hours. Eyestalks from crabs (15–18 mm carapace width) were dissected in saline (Cooke *et al.,* 1977) with the aid of a microscope. The entire optic tract including the major ganglia and sinus gland were removed from the eyestalk. These tissues were maintained in a 50 μl drop of saline that was periodically replaced, with the aid of a microliter syringe. The eyestalk was held in place with a suction electrode attached to the cut stump of the optic nerve. Electrical stimulation was given via the suction electrode in 40 ms duration pulses of various voltages (0.1, 0.5, 5, 10, 15, 20 volts). The order was randomized so the eyestalk did not receive a simple increasing stimulation. After a stimulation bout (2 minutes continuous voltage, 3 minutes rest) the saline perfusing the eyestalk was removed with a syringe and retained for bioassay. Voltage was delivered with a Phipps and Bird model 511 stimulator. Saline containing either a drug or neurotransmitter perfused the eyestalk for 5 minutes. The fluid was then removed with a syringe and retained for bioassay. The various concentrations of drugs or neurotransmitters were also presented in a random order. If both chemical and electrical stimulation were required the eyestalk was first perfused with the given drug or neurotransmitter for 5 minutes. Electrical stimulation was delivered to the tissue still in the saline containing drug or neurotransmitter. After the 3 minute rest the saline was removed for bioassay. The eyestalk was washed with fresh saline for 5 minutes between each electrical or chemical stimulation. An individual eyestalk was maintained *in vitro* for no more than two hours.

Perfusates were kept at 4°C until bioassayed. *Uca pugilator* exhibits a circadian rhythm of chromatophoric pigment dispersion and concentration. The pigments are more dispersed by day than at night. All eyestalks were dissected and stimulated between 0900 and 1130 hours. This ensured that some pigment dispersing endocrines were still stored in the sinus gland, which are reduced and replenished daily as a consequence of this rhythm (Fingerman and Fingerman, 1977b). All bioassays using

isolated legs were performed in the afternoon with legs from either eyestalk ablated crabs or crabs adapted to a black background (Herman and Dallmann, 1975; Hanumante and Fingerman, 1981a, b; Quackenbush, 1981). Eyestalkless crabs have maximally concentrated black and red pigments and provided the legs for the bioassay for pigment dispersing hormones. Intact crabs adapted to a black background have maximally dispersed black and red pigments and were used in bioassays for pigment concentrating hormones. An aliquot of each perfusate was carefully injected into 3 isolated legs (5 μl/leg) to avoid tissue damage. The black and red chromatophores were staged using the method of Hogben and Slome (1931) wherein stage 1 represents maximal pigment concentration, stage 5 maximal dispersion, and stages 2, 3, and 4 the intermediate conditions. The chromatophores were staged at the time of injection and 15, 30, 45, and 60 minutes thereafter. Isolated legs remain responsive to physiological doses of hormone for at least 60 minutes after removal from the crab (Herman and Dallmann, 1975; Quackenbush, 1981). Activity of the perfusates was determined using a modification of the Standard Integrated Response (SIR) technique of Fingerman *et al.* (1967). The activity values presented herein were calculated as follows. In assays for pigment dispersing actvity the sum all of the chromatophore stages determined at 0, 15, 30, 45, and 60 minutes with three legs given a control perfusate was subtracted from the sum of all the corresponding stages for three legs injected with perfusate from a stimulated eyestalk. The difference is the activity value. For example, if no change occurs in controls (3, +3 +3 +3 +3 = 15) and a maximum dispersion occurs in experimentals (3, +15 +15 +15 +15 = 63) the activity would be 48 (63 − 15), a maximal, possible response. On the other hand, to determine pigment concentrating activity the sum of the stages of isolated legs given a perfusate from a stimulated eyestalk was subtracted from the sum for the isolated legs given a control perfusate. Thus activity is the difference between the responses of the experimental and control legs. The activity values from the replicate experiments were averaged and standard deviations were calculated. Chromatophore stages are quantal data that cannot fulfill the requirements for parametric statistical analysis. Therefore, the conventional nonparametric substitute for the standard *t*-test, the Wilcoxon Rank Sum (WRS), was used to test differences between treatments and controls (Sokal and Rohlf, 1969).

The drugs were dissolved in isosmotic saline, 850 mOS, which had been aerated for one half hour prior to use. These solutions were prepared initially at 10^{-4} M and then serially diluted to achieve the desired doses. All drug solutions were prepared daily from dry frozen stocks. Norepinephrine hydrochloride and dopamine hydrochloride were purchased from Sigma Chemical Corp. Bretylium tosylate (Lot 177-36) was a gift of American Critical Care. Spiperone (lot A3301) was a gift of Janssen Pharmaceutica. In one set of experiments, saline with spiperone (10^{-5} M or 10^{-4} M) was injected into intact crabs (0.05 ml/crab). The crabs were then transferred from a black pan to a white pan in order to induce pigment concentration. This experiment tested whether spiperone, a dopamine antagonist, could block pigment concentration in intact crabs.

A small column of Sephadex G-25 was equilibrated with 50 mM phosphate buffer (pH 7.2). The void volume of the column (225 μl) was determined with blue dextran. The column was calibrated with Cytochrome C (12,400 d) and Bacitracin (1411 d). Perfusates from a single isolated eyestalk stimulated with 10 volts were pooled, lyophilized, then resuspended in 50 μl of the phosphate buffer (pH 7.2), applied to the top of the column, and eluted with the same buffer. Fractions of 25 μl were collected, adjusted to 850 mOS with 400% saline (Cooke *et al.*, 1977), and then bioassayed. The retention factor, R_f, was calculated by the formula: Void volume ÷ Elution volume. Protein was determined by the method of Peterson (1977). Synthetic RPCH

was purchased from Peninsula Laboratory (Belmont, California) and run on the Sephadex column as a standard.

RESULTS

Isolated eyestalks electrically stimulated

Eyestalks stimulated with 10 volts or more released substances which induced significant black pigment dispersion in isolated crab legs ($P < 0.05$, WRS test) (Fig. 1). No significant red pigment dispersing activity was detected after voltage stimulation (0.1 to 20 volts). Perfusates of eyestalks stimulated at 15 volts had significantly more black pigment dispersing hormone (BPDH) activity than those given 10 volts ($P < 0.05$, WRS test). Black pigment concentrating hormone (BPCH) activity was detected in perfusates of eyestalks given 0.5 volt; at 5 volts maximum BPCH activity was observed ($P < 0.05$, WRS test). RPCH activity was found in perfusates of eyestalks stimulated with 0.1 volt ($P < 0.05$, WRS test). The apparently different thresholds for the release of BPDH and BPCH may have been due to the antagonistic actions of these two peptides on the target cells. Perfusates of eyestalks stimulated with 5 volts had no significant BPDH activity (Fig. 2). However, perfusates from eyestalks stimulated with 10 volts had significant BPDH activity and the BPCH activity was clearly antagonized by BPDH, as seen by the drop of BPCH activity in Figure 1, and Figure 2. RPCH activity was not antagonized by red pigment dispersing hormone (RPDH) in perfusates from eyestalks stimulated with 5 or 10 volts (Fig. 3). Either RPCH completely antagonizes the action of any RPDH that might have been released, or no RPDH was released with this stimulation.

The data presented in Figure 1 demonstrate that the eyestalk releases several different factors when stimulated with 10 volts with this protocol. The isolated eyestalk may release only totipotent hormones which affect all chromatophores equally, as
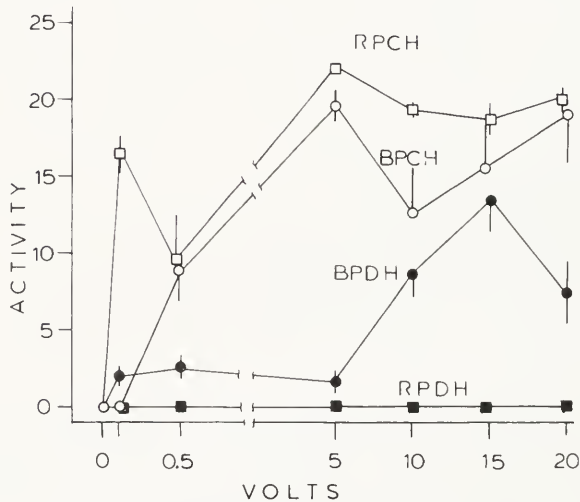

FIGURE 1. Activity of perfusates from isolated eyestalks stimulated with various voltages *in vitro.* RPDH activity (filled squares), RPCH activity (open squares), BPCH activity (open circles), and BPDH activity (filled circles) are compared to saline controls from unstimulated perfusates. Each point is the mean ± one standard deviation for 21 isolated legs given stimulated perfusate compared to 21 isolated legs given control perfusate (seven replicates).
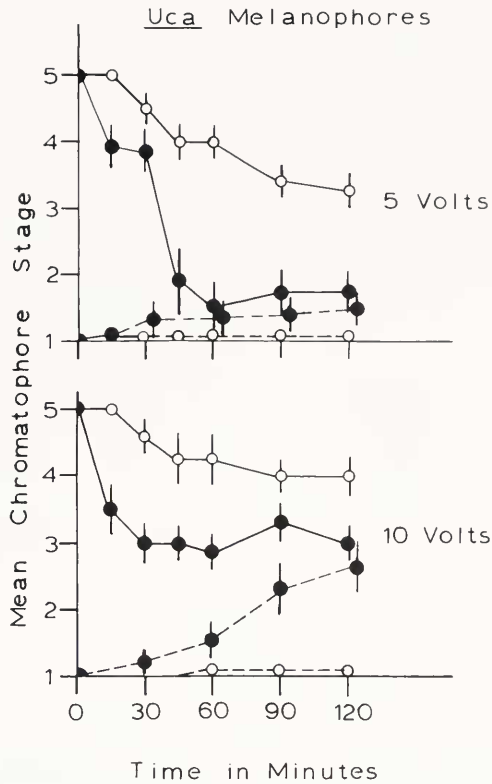
FIGURE 2. Mean chromatophore stages of isolated legs given perfusates from isolated eyestalks stimulated with 5 volts or 10 volts (solid circles) compared to isolated legs given perfusates from unstimulated isolated eyestalks (open circles). Solid lines connect the values for isolated legs with initially fully dispersed black pigment. Dashed lines connect the values for isolated legs with initially fully concentrated black pigment. All values are means ± one standard devation (n = 21, seven replicates).

proposed by Josefsson (1983), or the eyestalk may release hormones specific for each type of chromatophore. One way to distinguish among these alternatives would be to separate the chromatophorotropins in the perfusates. Therefore we chromatographed pooled perfusates from 10 volt stimulation bouts to determine if totipotent peptides or peptides with target cell specificity were being released. Assays were performed for BPDH, BPCH, RPCH, and RPDH activities in each fraction after the void volume.

Red pigment concentrating activity was found in 4 different fractions (R_f mean ± one standard deviation for 4 replicates; 0.83 ± 0.02; 0.65 ± 0.03; 0.50 ± 0.05; and 0.34 ± 0.02) (Fig. 4A). The fraction at R_f 0.50 had 0.9 ± 0.3 pg protein, and co-chromatographed with BPDH activity. This fraction (0.50 ± 0.02) had no BPCH activity. The fraction at R_f 0.34 contained a significant amount of RPCH activity ($P < 0.05$, WRS test) which co-chromatographed with the synthetic RPCH. The *Uca* material at R_f 0.34 had no other activity in these assays. The fraction at R_f 0.625 had both BPCH and RPCH activity, at slightly different levels. RPDH activity was not detected in any fractions assayed. We see that stimulation of the isolated *Uca* eyestalk results in the release of several different compounds, some sharing biological activity while others induce a specific target cell response.

Substances with BPCH activity were found in three fractions (R_f 0.63 ± 0.02;
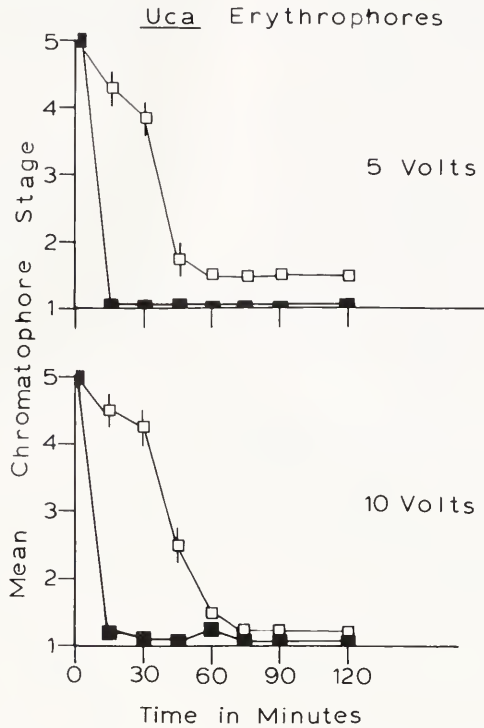
FIGURE 3. Mean chromatophore stage of isolated legs given perfusates from isolated eyestalks stimulated with 5 volts or 10 volts (solid squares) compared to isolated legs given perfusates from unstimulated eyestalks (open squares). Solid lines connect the values from isolated legs with fully dispersed red pigment. Isolated legs with initially fully concentrated red pigment were also tested, but showed no effect of perfusate injection (data not shown). All values are means ± one standard deviation (n = 21, seven replicates).

0.43 ± 0.03; and 0.38 ± 0.01). Two different fractions had peptides (0.6 ± 0.02 pg protein for each fraction) in the low molecular weight range (less than 5,000 d) that only affected black chromatophores. BPDH activity was resolved into 3 fractions after the void volume (R_f 0.50 ± 0.02 had 0.9 ± 0.3 pg protein, 0.75 ± 0.02 and 0.41 ± 0.01 each had 0.4 ± 0.15 pg protein). It should be noted that two fractions containing BPDH had no BPCH activity. The presence of several sizes of peptides which have biological activity in these perfusates suggests that processing of precursors may occur within the eyestalk complex. Furthermore, the isolated eyestalk complex releases these large sized compounds when stimulated with voltage. Finally, the eyestalk complex contains large compounds which can affect two different target cells, as well as different compounds which have activity on only one target cell.

Isolated eyestalks stimulated with NE

Isolated eyestalks were bathed in saline to which one of six different NE concentrations was added (10^{-11}–10^{-6} M) (Fig. 5). Exogenous NE did not induce the release of any substance which produced either significant dispersion or concentration of the red pigment. Isolated eyestalks in saline with 10^{-11} M NE released BPDH in detectable
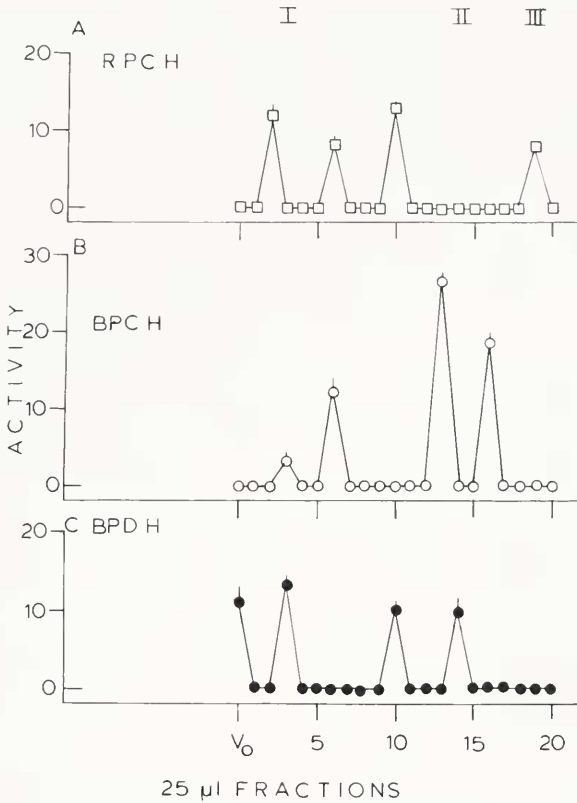
FIGURE 4. Activity of fractions eluted from a Sephadex G-25 column of the perfusate of isolated eyestalks (n = 4) stimulated with 10 volts on isolated legs. RPCH (panel A) activity is indicated by open squares. BPCH (panel B) activity is indicated by open circles. BPDH (panel C) activity is indicated by solid circles. Each activity value is the mean ± one standard deviation for 12 legs (four replicates). Numeral I indicates where Cytochrome C (12,400 d) eluted; II indicates where Bacitracin (1,411 d) eluted; and III where synthetic RPCH eluted (n = 12, four replicates).

quantities ($P < 0.01$, WRS test). The dose response curve (Fig. 5) of the effect of exogenous NE on isolated eyestalks reveals that the most BPDH was released in response to 10^{-9} M NE. Increasing the NE concentration above 10^{-9} M significantly decreased the level of BPDH in the perfusates (WRS test $P < 0.05$).

In a separate series of experiments isolated eyestalks were perfused with 10^{-6} M NE, then stimulated with 10 volts. Stimulation with both 10^{-6} M NE and 10 volts resulted in significantly more BPDH in the perfusate than stimulation with 10^{-6} M NE alone or 10 volts alone ($P < 0.01$, WRS test, Fig. 6). As expected, no significant red pigment dispersion was detected in perfusates from any combination of stimulation with volts and NE. Isolated eyestalks given bretylium (2×10^{-4} M), a blocker of impulse mediated NE release (Boura and Green, 1959), and subsequently stimulated with 10 volts released no detectable BPDH into the perfusate (Fig. 6). Since the chromatophores on isolated legs are unaffected by NE or bretylium (Fingerman *et al.*, 1981a, b; Hanumante and Fingerman, 1981a, b; 1982a, b) we conclude that NE can induce the release of BPDH from the isolated eyestalk.
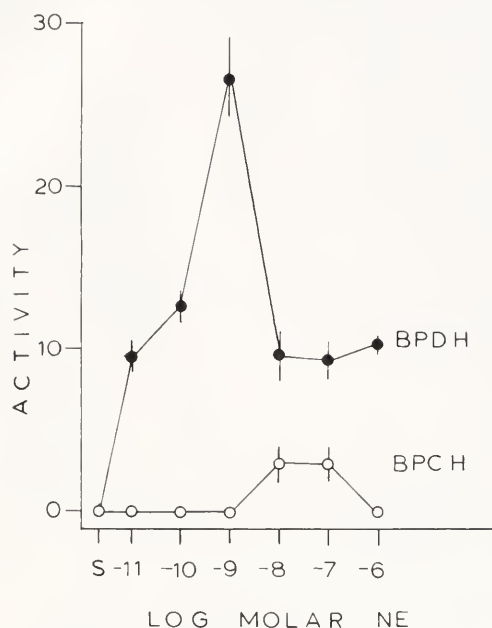
FIGURE 5. Dose response curve for the effect of NE on the biological activity of the perfusate of isolated eyestalks in isolated legs. BPDH activity is represented by solid circles and BPCH activity is represented by open circles. Each value is the mean ± one standard devation (n = 27, nine replicates). Saline value indicated by S.

Isolated eyestalks stimulated with DA

DA produces red pigment concentration when injected into whole crabs, but it has no effect on the chromatophores of isolated legs (Fingerman and Fingerman,
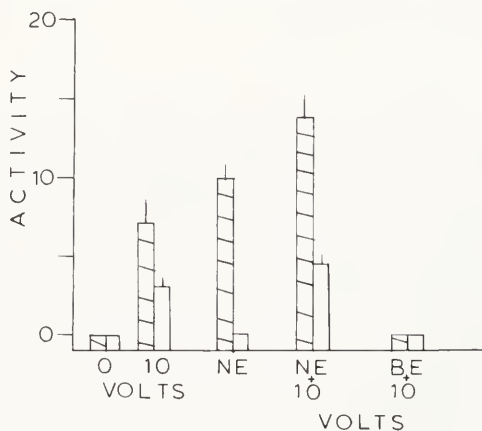

FIGURE 6. The effect of NE (10^{-6} M) and voltage stimulation (10 volts) on BPDH and RPDH activity in perfusates from isolated eyestalks. Open bars are values for red pigment and hatched bars are values for black pigment dispersion in isolated legs given perfusates from isolated eyestalks treated as indicated. Bretylium (2×10^{-4} M) was added to saline which perfused eyestalks prior to voltage (10 volt) stimulation indicated by BE + 10. All values are means ± one standard deviation (n = 18, six replicates).

1977a; Fingerman *et al.*, 1981a). This exogenous DA presumably exerts its effect by triggering RPCH release. The site of action of the DA could be the eyestalk or the central nervous organs within the body proper such as the supraesophageal ganglia. The crustacean central nervous system within the body proper not only contains RPCH (Brown, 1973) but also DA (Eloffson *et al.*, 1982).

Isolated eyestalks were bathed in 8 concentrations of DA (10^{-12}–10^{-5} M). At all but the weakest dose significant, amounts of RPCH and BPCH were detected in the bathing media (Fig. 7) ($P < 0.05$, WRS test). Although DA did not induce black pigment concentration *in vivo* (Fingerman and Fingerman, 1977a), significantly more BPCH than RPCH was found in the perfusates (WRS $P < 0.01$, 10^{-10}, 10^{-9}, 10^{-6}) when tested *in vitro*. DA has no direct action on either type of chromatophore when injected into isolated legs.

In order to add support to the new observation that DA can mediate the release of BPCH, the well known mammalian DA antagonist, spiperone, was used in *in vivo* and *in vitro* experiments. In intact whole crabs given 10^{-5} or 10^{-4} M spiperone, concentration of the black pigment was blocked but red pigment concentration was unaffected (Fig. 8). When isolated *Uca* eyestalks were stimulated with 5 volts the perfusate contained both RPCH and BPCH (Fig. 1). We then used a 5 volt stimulation of isolated eyestalks bathed in saline with spiperone to determine the effect *in vitro*. Perfusates from isolated eyestalks stimulated with 5 volts in saline with spiperone at 10^{-9} M had no BPCH but did have RPCH (Fig. 9). At higher concentrations of spiperone only BPCH was absent from the perfusate. Spiperone may act on the
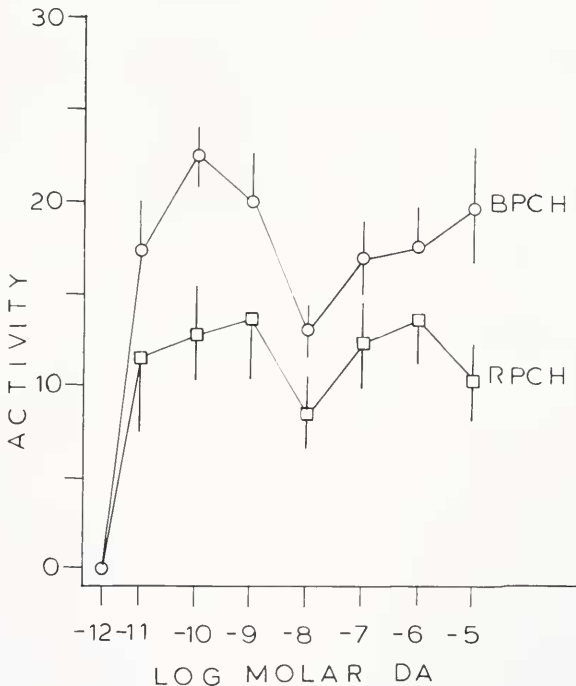

FIGURE 7. Dose response curve for the effect of DA on the biological activity of the perfusate of isolated eyestalks. The assays were performed on isolated legs. RPCH activity is represented by open squares and BPCH activity is represented by open circles. Each value is the mean ± one standard deviation (n = 27, nine replicates).
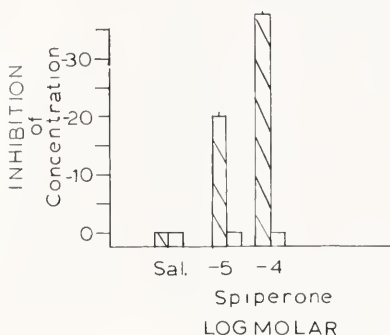
FIGURE 8. The effect of spiperone on pigment concentration in intact crabs which were changed from a black background to a white background. Open bars are values for red pigment and hatched bars are values for black pigment. Control crabs concentrate their black and red pigments when this background change experiment is performed. Activity was calculated as described in Methods, negative values indicate inhibition. For this experiment activity values were based on 5 crabs given treatments compared to 5 crabs as control. Saline values are indicated by Sal, spiperone in saline at 10^{-5} M values by 5 and spiperone in saline at 10^{-4} M values by 4. All values are means ± one standard deviation (n = 30 each group, six replicates).

eyestalk to produce extraordinarily large BPDH release, so an additional experiment was done. Eyestalks were bathed in saline containing spiperone then stimulated with 10 volts. This perfusate was then passed through the small Sephadex column and BPDH, RPCH, and BPCH bioassays were done on each fraction (Table I). BPDH activity was not affected by spiperone. Perfusates with spiperone had significantly more of the smallest RPCH than the control perfusates though no effect on the RPCH's of two other sizes were detected. Spiperone at 10^{-6} M completely blocked
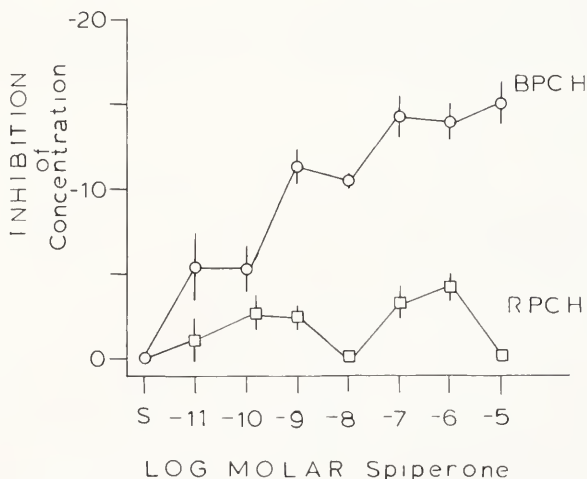
FIGURE 9. Dose response curve for the effect of spiperone in saline bathing isolated eyestalks stimulated with 5 volts on the presence of BPCH and RPCH measured in isolated legs. BPCH (open circles) and RPCH (open squares) activity was determined as described, negative values indicate inhibition. Increasing values mean increasing inhibition of BPCH or RPCH release as compared to controls of saline perfused eyestalks stimulated with 5 volts. Each value is the mean ± one standard deviation (n = 27, nine replicates). Saline bathing eyestalks stimulated with 5 volts (control) indicated by S.

the voltage induced release of all forms of the BPCH expected with a 10 volt stimulation (Table I).

DISCUSSION

The control of pigment migration in integumental chromatophores of the fiddler crab *Uca pugilator* by antagonistic peptide hormones presents a difficult challenge to the neuroendocrine system. The hormones we studied are all produced in and released from the same neurohemal complex within the eyestalk. However, crabs can maintain the pigment in each type of chromatophore in different stage of dispersion so the black pigment may be fully dispersed while the red pigment is fully concentrated. Therefore, some mechanism for the independent release of specific chromatophorotropic hormones may exist.

Stimulation of the isolated *Uca* eyestalk with different voltages apparently differentially releases RPCH, BPCH, and BPDH. This stimulation may act upon neurosecretory cells directly or on neurons presynaptic to the neurosecretory cells. The apparently high threshold for BPDH release may be a result of the presence of its antagonist BPCH. Similarly, the low threshold of RPCH release may mask the presence of RPDH which was not detected in any of our experiments. Thus regulation of chromatophore stage in crabs could be the result of different levels of the antagonistic pairs of hormones acting on the target cells (Rao and Fingerman, 1983). This is supported by the comparison of biological activities of the components of the pooled 10 volt perfusate before and after separation by gel filtration chromatography (Figs. 1 and 4). The total BPCH activity increased after separation from its antagonist, BPDH. The increase of total activity of the RPCH after chromatography also argues for the presence of RPDH, though it was not detected.

Electrical stimulation of the isolated eyestalk triggers release of peptides which only affect one target cell (Fig. 4). This contradicts the observation that crustaceans use peptides which are totipotent (affecting both melanophores and erythrophores) as the primary regulators of color change (Fernlund and Josefsson, 1972; Quackenbush, 1981; Josefsson, 1983). Previous results using catecholamines and 5-HT to trigger release of chromatophorotropins suggested each neurotransmitter had a specific effect that could not be a result of the release of a totipotent peptide (Rao and Fingerman,

TABLE I

Effect of spiperone (10^{-6} M) on voltage stimulated release of BPDH, RPCH, and BPCH activity from isolated eyestalks

		Activity (mean ± one standard deviation)		
	R_f	10 volts	10 volts + 10^{-6} M spiperone	Significance
BPDH	0.76	13.55 ± 1.1	12.55 ± 0.89	NS
	0.50	10.36 ± 1.5	10.1 ± 1.1	NS
RPCH	0.625	8.8 ± 0.89	10.3 ± 1.7	NS
	0.50	13.2 ± 0.68	13.0 ± 0.68	NS
	0.34	8.2 ± 0.73	23.0 ± 1.18	$P < 0.01$
BPCH	0.625	12.5 ± 1.5	0	$P < 0.001$
	0.43	26.0 ± 1.09	0	$P < 0.001$
	0.38	18.5 ± 1.10	0	$P < 0.001$

1970; Fingerman and Fingerman, 1975; 1977a, b; Fingerman *et al.*, 1981a, b). Those experiments with intact crabs did report that the brain was one site for the action of the neurotransmitter, though the eyestalk was also a likely target. The present *in vitro* study confirmed *in vivo* studies which demonstrated that NE only induces BPDH release (Fingerman and Fingerman, 1977; Fingerman *et al.*, 1981). Bretylium blocked voltage induced release of BPDH, which suggests that voltage stimulation acted through a pathway involving NE release. High (10^{-6} M) levels of NE released significantly less BPDH than lower doses (10^{-9} M). This has been observed previously with neurotransmitter induced release of vertebrate peptides (Koch, 1970; Bower *et al.*, 1974; Hadley *et al.*, 1975). A negative feedback role for the neurotransmitter was postulated. Hanumante and Fingerman (1982b) have already postulated a negative feedback mechanism for NE regulation of BPDH based on pharmacological experiments *in vivo*. The observation here that voltage stimulation can augment 10^{-6} M NE induced BPDH release provides additional support for this hypothesis.

Fingerman and Fingerman (1977a) found 2.6×10^{-3} M DA induced RPCH but not BPCH release in whole crabs maintained on a black background. In contrast, BPCH release by DA was apparent in experiments with isolated eyestalks and isolated legs (Fig. 7). The crabs were presumably releasing BPDH into their blood to keep the black pigment dispersed in response to that background. In intact crabs circulating BPDH could have antagonized and masked any released BPCH. In the *in vitro* experiments there was presumably insufficient BPDH to mask all of the BPCH in the injected perfusate. We now find that dopamine can induce both RPCH and BPCH release *in vitro*. However, spiperone, in both *in vivo* and *in vitro* assays only affects the release of BPCH, not RPCH. Clearly, there must be a further subdivision of DA stimulation of peptide release in the isolated eyestalk, perhaps similar to the mammalian dopamine$_1$ and dopamine$_2$ systems (Lichtensteiger, 1979).

The actual mechanisms by which the crustacean sinus gland controls the release of specific hormones is not known. This neurohemal organ stores several distinct hormones, and some produce antagonistic actions in the crab (*e.g.*, BPDH and BPCH). The voltage dose response curves (Fig. 1) suggest such differential thresholds for endocrine release may provide an experimental approach for studying the release of target cell specific hormones. A physiological mechanism may be the specific release of a neurotransmitter inducing release of a specific hormone from a peptidergic neuron. Clearly NE has a singular action, inducing BPDH release.

The demonstration here and by others (Cooke *et al.*, 1977; Newcomb, 1982) that the crustacean eyestalk neuroendocrine system releases several peptides of different sizes suggests that these peptides are processed from larger precursors. The demonstration of large and small peptides with the same biological activities (Fig. 4) supports the notion that processing into smaller peptides can occur at the sinus gland.

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